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Phosphoinositide-dependent kinase 1 targets protein kinase A in a pathway that regulates interleukin 4

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CD28 plays a critical role in T cell immune responses. Although the kinase Akt has been shown to act downstream of CD28 in T helper (Th)1 cytokine induction, it does not induce Th2 cytokines such as interleukin 4 (IL-4). We recently reported that phosphoinositide-dependent kinase 1 (PDK1) partially corrects the defect in IL-4 production present in CD28-deficient T cells, suggesting that PDK1 regulates IL-4 independently of Akt. We now describe a signaling pathway in which PDK1 targets IL-4 in the murine Th2 cell line D10. PDK1-mediated activation of this pathway is dependent on protein kinase A (PKA) and the nuclear factor of activated T cells (NFAT) P1 transcriptional element in the IL-4 promoter. PDK1 localizes to the immune synapse in a phosphatidylinositol 3-kinase-dependent manner, partially colocalizes with PKA at the synapse, and physically interacts with PKA. In RNA interference knockdown experiments, PDK1 is necessary for phosphorylation of PKA in T cells, as well as for activation of the IL-4 NFAT P1 element by the T cell receptor (TCR) and CD28. Phosphorylation of the critical PKA threonine residue is stimulated by engagement of TCR/CD28 via a PDK1-dependent mechanism. These findings together define a pathway linking the kinases PDK1 and PKA in the induction of the Th2 cytokine IL-4.

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Abbreviations used: AKAP, A-kinase anchoring protein; CRE, cAMP-responsive element; DN, dominant negative; KH, kinase hyperactive; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; PIF, PDK-interacting fragment; PKA, protein kinase A; PKAc, PKA catalytic subunit; RNAi, RNA interference; siRNA, small interfering RNA.

T cells play a critical role in normal immune responses and in the pathogenesis of many autoimmune diseases. T cells are normally activated by signals mediated by the antigen receptor and by costimulatory molecules, the best characterized of which is the CD28 surface receptor, which binds to CD80 and CD86 receptors on APCs (1). CD28 plays a critical role in the normal expression of Th1 and Th2 cytokines (2, 3), with CD28-deficient cells demonstrating a profound deficiency in expression of multiple cytokines (4, 5). Th2 cytokines have been shown to play a pivotal role in the pathogenesis of allergic diseases such as asthma (6). Cytokine-dependent signaling pathways that mediate both Th1 and Th2 cytokine production are well characterized with Th2 differentiation controlled by IL-4, STAT6, and the downstream transcription factors c-maf and GATA-3 (7–9). The pathways downstream of CD28 and other costimulatory molecules that are required for production of Th2 cytokines are, however, less well understood.

Previous work from our group has shown that the serine-threonine kinase Akt can correct the deficiency in Th1 cytokine production exhibited by CD28-deficient T cells (5). Interestingly, however, Akt had no apparent effect on the production of Th2 cytokines. This result suggested the existence of an alternative pathway downstream of CD28 that regulates Th2 cytokines. The activation of Akt is regulated by the upstream serine-threonine phosphoinositide-dependent kinase 1 (PDK1) (10). Recently, we have shown that expression of an activated form of PDK1, designated PDK1-kinase hyperactive (KH), partially corrects the deficiency of Th2 cytokine production in CD28-deficient cells (11). This isoform of PDK1 contains a point mutation in the kinase domain (A277V) that results in a three-fold increase in kinase activity (12). Two types of signaling motifs in the CD28 cytoplasmic tail have been defined as critical for Th2 cytokine expression in primary CD4⁺ T cells (11). Tyrosine residues that undergo inducible phosphorylation represent the first such motif. Mutation of tyrosine residues 170 and 188 in CD28 results in defective Th2 cytokine

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induction (11). Of note, the Y170 residue has been previously shown to bind the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and Grb-2 (13–15). A proline-rich region in the CD28 tail (16) represents a second motif required for Th2 cytokine expression (11), as well as a potential binding site for proteins containing SH3 domains. Although expression of PDK1-KH alone did not autonomously correct Th2 cytokine defects in CD28-deficient primary T cells, coexpression with CD28 constructs mutated in either of these motifs corrected their defect in Th2 cytokine production (11). Coexpression of Akt was unable to similarly complement the CD28 mutant proteins and restore Th2 cytokine production. As such, these signaling motifs in the CD28 cytoplasmic tail may act to generate signals that synergize with PDK1 to promote Th2 cytokine expression in primary T cells. In addition to effects on Th2 cytokine expression, PDK1-KH was able to autonomously restore Th1 cytokine production in CD28-deficient cells in a similar fashion to membrane-targeted Akt (11). This result suggests that, in addition to playing a role in Th2 cytokine production, PDK1 acts upstream of Akt in Th1 cytokine signaling pathways.

PDK1 is a ubiquitously expressed 67-kD serine-threonine kinase containing an amino-terminal kinase domain and a carboxy-terminal PH domain (10). PDK1 plays an important role in T cell thymic development (17), but its role in peripheral T cell function is unknown. Although PDK1 was originally identified for its ability to phosphorylate Akt on a critical functional residue (18), it has also been shown to activate several other serine-threonine kinases, including PKC isozymes, p70S6K, RSK, SGK, and cAMP-dependent protein kinase A (PKA) (10, 19–22). Akt contains a PH domain that mediates PIP3 binding and consequently colocalization with PDK1 (23). Other substrates, notably PKC, RSK, and p70S6K, contain a so-called PDK-interacting fragment (PIF) that mediates interaction with and facilitates phosphorylation by PDK1 (19, 24). Structural analysis has revealed the presence of a hydrophobic region within the PDK1 kinase domain termed the “PIF pocket” that mediates interaction with the PIF domain (25). Gene knockout and “knockin” studies of PDK1 have provided several new insights into its function. Mice with deletion of the PDK1 gene or with knockin mutations in the PH domain or the PIF pocket all die by embryonic day 9.5, reinforcing the essential role of the kinase (26–28).

We originally hypothesized a role for PDK1 in CD28 signaling as a result of the defined role of its substrate Akt in costimulation-dependent Th1 cytokine activation. Elevation of intracellular cAMP levels and activation of PKA have previously been shown to positively impact Th2 differentiation and IL-4 production (29–32). The mechanisms whereby PKA modulates Th2 cytokine expression remain incompletely characterized. Other studies have demonstrated that PDK1 phosphorylates PKA at a critical threonine residue in its kinase domain *in vitro* (21, 22). We had hypothesized that PDK1 may mediate a distinct Th2 signaling pathway by activating PKA and now present data supporting this hypothesis. We demonstrate that PDK1 directly targets the IL-4 pro-

motor in the antigen-dependent murine Th2 cell line D10.4G1 (D10) (29) and that PKA acts downstream of PDK1 in this pathway, which targets the NFAT P1 element in the IL-4 promoter. We show that the essential threonine residue of PKA is inducibly phosphorylated after CD3/CD28 engagement. Using RNA interference (RNAi) technology,

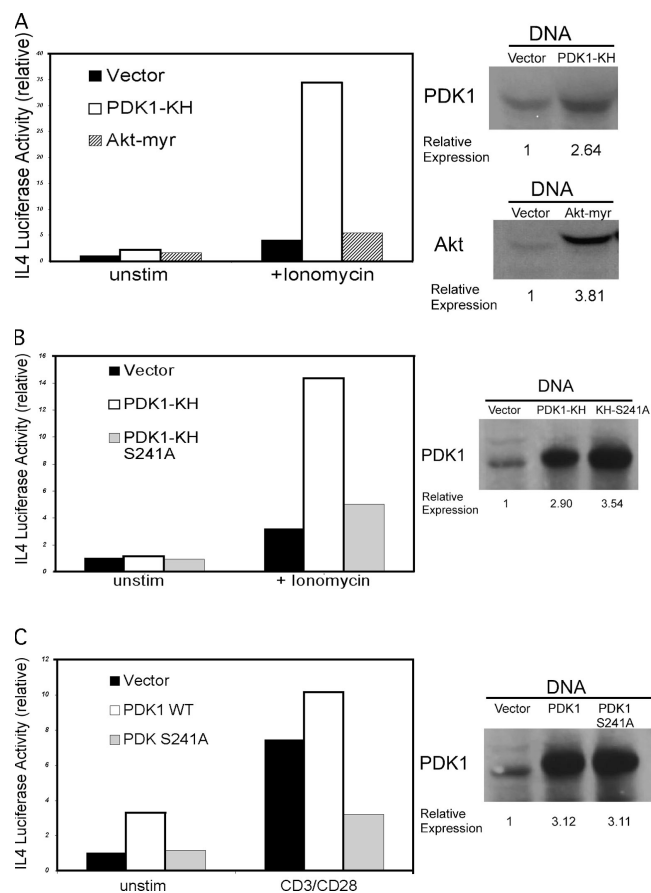


Figure 1. PDK1 but not Akt activates the IL-4 promoter. (A) PDK1-KH or a myristoylated membrane targeted form of Akt (Akt myr) was co-transfected with an IL-4 –800 Luciferase transcriptional reporter in the D10 cell line. 16 h later, 10^5 cells were either left unstimulated or were treated with ionomycin ($0.5 \mu\text{M}$) and were assayed for luciferase activity 6 h later. Luciferase data are shown relative to unstimulated IL-4 (or other reporter) luciferase activity (relative activity = 1) throughout the paper unless otherwise stated. A representative experiment is shown with all such experiments shown in this manuscript repeated at least three times. The mean result for each sample (performed in duplicate) is shown. Western blot expression controls were performed using 10^6 remaining cells with transfected PDK1-KH and Akt-myr compared with endogenous protein with quantification of fold-overexpression. (B) PDK1 kinase activity is required for IL-4 promoter activation. PDK1-KH and a PDK1-KH construct with a kinase-inactivating S241A mutation were coexpressed with IL-4 Luciferase. Western blot expression controls are shown. (C) PDK1-DN (S241A) blocks CD3/CD28-mediated activation of the IL-4 promoter. Wild-type PDK1 or PDK1-DN were coexpressed with the IL-4 Luciferase reporter. After 16 h, cells were stimulated with plate-coated anti-mouse CD3 antibody with soluble mouse anti-CD28 (1:500 in media) or were placed in a control plate (unstim). Protein expression controls are shown.

PDK1 expression is shown to be required in D10 T cells for maximal basal and inducible phosphorylation of PKA, as well as for activation of the IL-4 NFAT P1 transcriptional element. We also show that PDK1 and the PKA catalytic subunit physically interact, and that these kinases colocalize at the immune synapse. These studies link PDK-1 and cAMP-dependent PKA in a pathway targeting IL-4 expression.

RESULTS

PDK1 activates the IL-4 promoter in D10 T cells

As discussed in the Introduction, we have previously reported studies defining a role for PDK1 in Th2 cytokine production in primary T cells. We have now studied the effects of PDK1 on IL-4 promoter activity in the D10 Th2 cell line. We initially observed that transfection of PDK1-KH potentially increased IL-4 promoter activity in D10 cells (Fig. 1 A). Maximal IL-4 promoter activation required the presence of the calcium ionophore ionomycin to simulate calcium-dependent signals emanating from the antigen receptor. In contrast, transfection of a membrane-targeted form of the PDK1 substrate Akt (Akt-myr) had minimal effects on the IL-4 promoter under these conditions. Western blot analysis of

PDK1-KH and Akt-myr levels compared with endogenous proteins showed similar levels of overexpression for each protein. We next tested whether activation of IL-4 required PDK1 kinase activity by introducing a previously described inactivating mutation (S241A) (33) into the PDK1 kinase domain. Introduction of this mutation into the PDK1-KH protein substantially reduced activation of the IL-4 promoter with both proteins overexpressed at similar levels (Fig. 1 B). Wild-type PDK1 containing the S241A mutation acted as a dominant negative (DN) isoform of PDK1 (PDK1-DN) because it was capable of blocking CD3/CD28-mediated activation of the IL-4 promoter (Fig. 1 C). In contrast, wild-type PDK1 expressed at similar levels did not block CD3/CD28 stimulation of IL-4 promoter activity. That PDK1-S241A was indeed acting as a specific DN isoform was confirmed by demonstrating that expression of this protein could block Akt phosphorylation by endogenous PDK1 after CD3/CD28 stimulation (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051715/DC1>). These results, together with our previous studies in primary CD28-deficient T cells, suggest that PDK1 may play a role in regulating the transcriptional activity of the IL-4 gene.

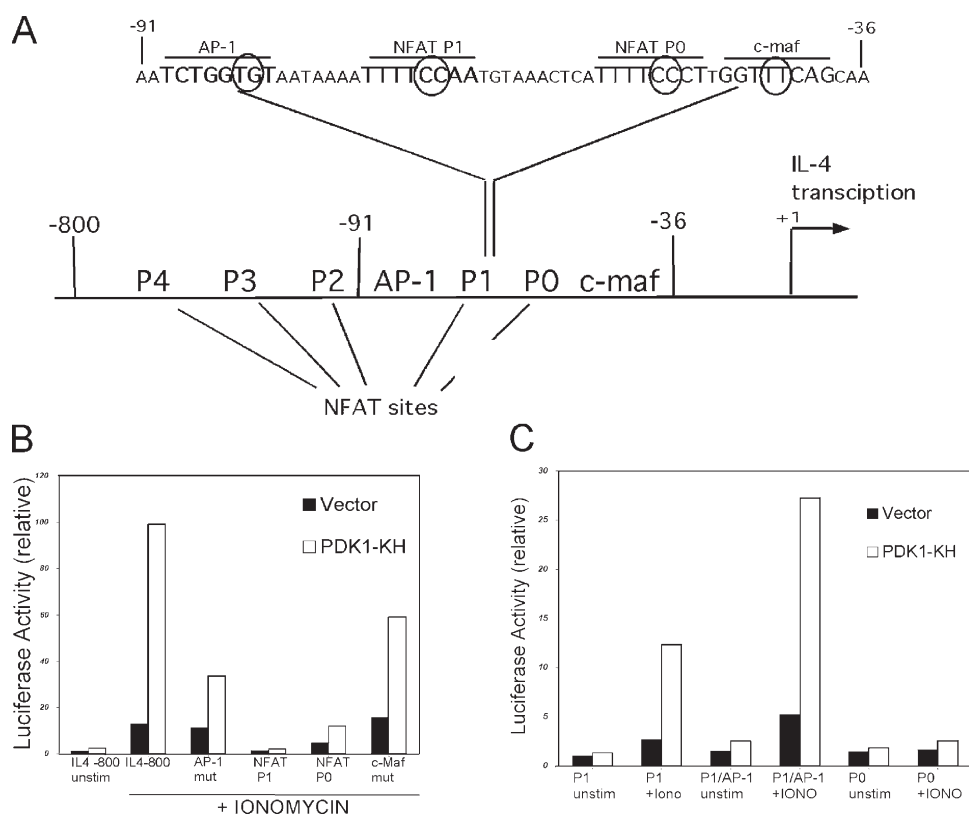


Figure 2. PDK1 targets the NFAT P1 site in the IL-4 promoter. (A) Schematic of the IL-4 promoter showing selected promoter elements and the sequence of the proximal promoter from -91 to -36. The elements studied in this analysis are highlighted with larger letters with mutated nucleotides circled. (B) PDK1-KH activation of IL-4 requires the NFAT P1 site. PDK1-KH was coexpressed with wild type or mutant IL-4

promoter constructs. Transfected D10 cells were treated with ionomycin 6 h before measurement of luciferase activity (except for samples labeled unstim). (C) PDK1-KH was coexpressed with multimerized 2X-reporter constructs for IL-4 NFAT P1, NFAT P1/AP-1, and NFAT P0. Cells were treated with ionomycin or left unstimulated as labeled and assayed for luciferase activity.

PDK1 targets the NFAT P1 element in the IL-4 promoter

We next mapped the transcriptional element required for activation of the IL-4 promoter by PDK1. The murine IL-4 luciferase construct mediating responsiveness to PDK1 contained ~740 base pairs of promoter sequence upstream of the transcription start site (34). We constructed deletion mutants of the promoter and found that the region spanning -91 to -36 of the promoter was required for activation of IL-4 by PDK1 (unpublished data). As shown in Fig. 2 A, this area of the promoter contains binding sites for NFAT and AP-1 transcription factor families (34, 35), as well as a binding site for the Th2-specific transcription factor c-maf (8), which have all been shown to be important for normal IL-4 regulation. Subsequent mutation of the NFAT P1 site, but not the NFAT P0 site or the c-maf and AP-1 binding sites, com-

pletely ablated activation of the IL-4 promoter by PDK1-KH (Fig. 2 B). As both NFAT sites in this region have been shown to be important for maximal IL-4 promoter activity (36, 37), it was unclear whether the NFAT P1 site is a specific target of PDK1 action or whether mutation of the site simply renders the promoter completely unresponsive to any stimuli. We confirmed that the NFAT P1 site is a target of PDK1 activation by creating reporter constructs with multimers of this site. Expression of PDK1-KH preferentially activated an NFAT P1 reporter construct, but did not significantly influence the activity of the IL-4 NFAT P0 reporter (Fig. 2 C). Creation of an NFAT P1 reporter with the neighboring AP-1 site (P1/AP-1) conferred increased responsiveness to ionomycin and PDK1. The NFAT P1 site may serve as a composite transcriptional element with the adjacent AP-1

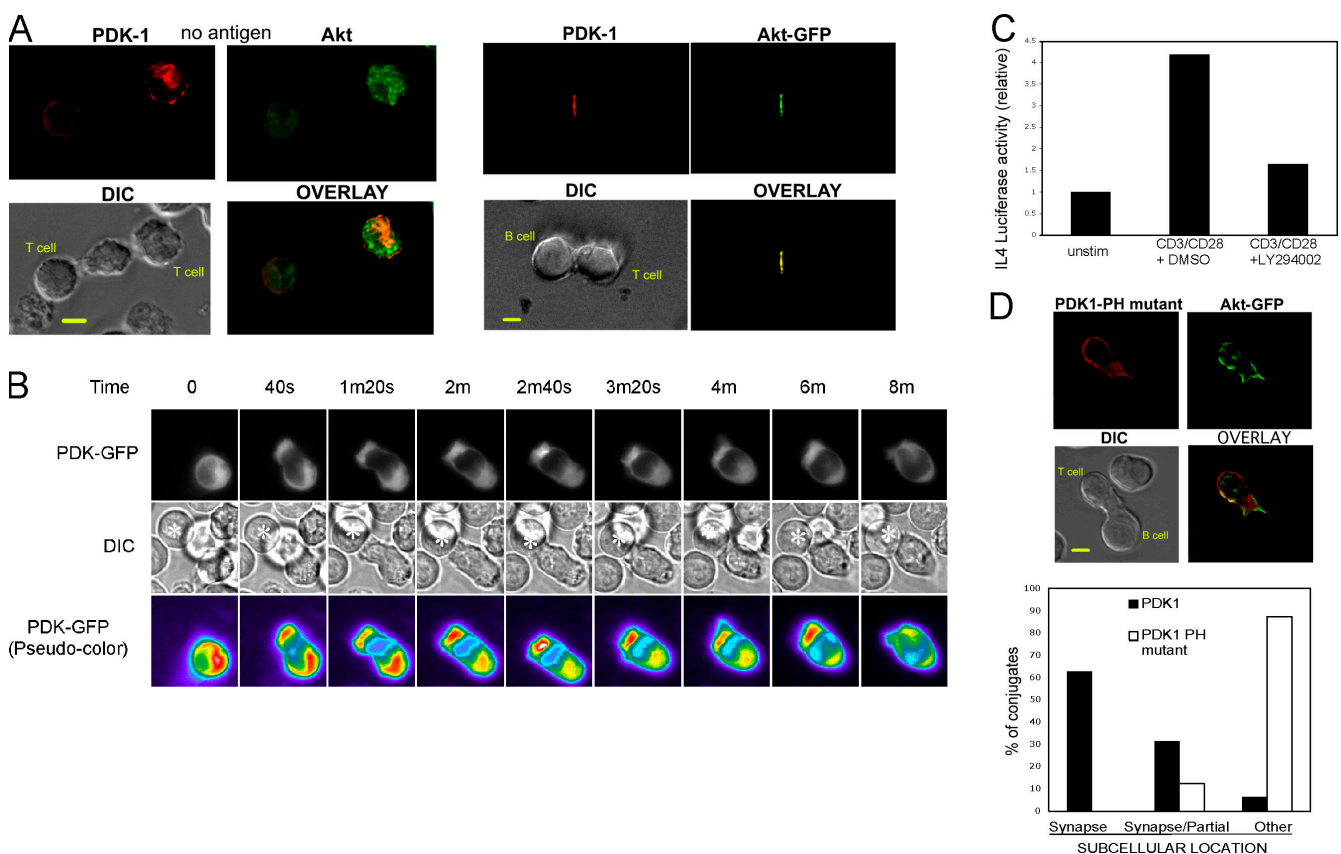


Figure 3. (A) PDK1 colocalizes with Akt at the immune synapse.

D10 cells were transfected with myc-tagged wild-type PDK1 and with an Akt-GFP fusion protein. Transfected cells were allowed to form conjugates with CH27 B cells in the presence or absence of antigen. Cells were subsequently fixed and stained with an anti-myc Cy3-conjugated antibody and visualized using a Zeiss deconvolution microscope. Bar, 5 μ m. (B) Kinetics of recruitment of PDK1 to the immune synapse. PDK1-GFP localization after conjugate formation between D10 T cells and conalbumin-loaded CH27 APCs. Green fluorescence (top), DIC (middle), and pseudo-colored images (bottom) were obtained every 20 s for 20 min with time-lapse fluorescence microscopy. Images are representative of several conjugates. *, APCs. (C) IL-4 promoter activation is sensitive to

the PI3K inhibitor LY294002. Cells were transfected with the IL-4 luciferase construct and either left unstimulated or stimulated with plate-bound anti-CD3 ϵ antibody and soluble anti-CD28 antibody in the presence of LY294002 (10 μ M) or DMSO control. (D) A PDK1 PH mutant (L472/474E) does not localize to the immune synapse. A representative micrograph is shown (top) for the PDK1 PH mutant in a D10-CH27 conjugate in the presence of antigen. Bar, 5 μ m. Blinded analysis of 20 conjugates (in two separate experiments) using wild-type PDK1 or the PDK1 PH mutant was performed. The mean number of conjugates in which the respective PDK1 protein localizes predominantly to the immune synapse, partially to the synapse, or to other subcellular locations is shown (bottom).

site as has been described for the distal IL-2 NFAT element (38). As PDK1 can activate NFAT P1 constructs lacking an AP-1 binding site, it likely has direct effects on the function of NFAT transcription factors. Enhanced activation by PDK1 of the composite P1/AP-1 reporter could be explained by distinct effects of PDK1 on AP-1 transcription factors or may simply reflect the well-established synergy between NFAT and AP-1 factors in composite transcriptional elements.

PDK1 localizes to the immune synapse

We were interested in examining the cellular localization of PDK1 in T cells because our results implicated PDK1 in CD28 signaling and IL-4 production. We again used the D10 cell line, which expresses a TCR specific for a conalbumin peptide in the context of the MHC class II Ia^k subtype (29). The CH27 B cell line serves as an effective APC in this system. We examined localization of PDK1 coexpressed with an Akt-GFP fusion protein. Akt has been recently shown to localize to the area at the T cell-APC interface, designated the immune synapse (39). We found that PDK1 is present mainly in the cytoplasm in resting T cells (Fig. 3 A, left) but translocates to the immune synapse after engagement by the APC (Fig. 3 A, right). Localization of PDK1 to the synapse was antigen-dependent and conjugates were not seen in the absence of conalbumin (Fig. 3 A, left). PDK-1 colocalized with Akt at the synapse in an antigen-dependent fashion (Fig. 3 A, right).

The kinetics of PDK1 localization to the immune synapse was examined using live cell imaging of a PDK1-GFP fusion protein. In the representative time course shown (Fig. 3 B), initial contact is noted between the transfected D10 T cell and the CH27 APC by the second panel at the 40-s time point. Definitive contact is established by the third panel at 2 min, with some immediate enrichment of PDK1 at the immune synapse. A large amount of PDK1 is also noted in the trailing edge (uropod) of the T cell, which has reproducibly been noted in cells transfected with moderate-to-high amounts of PDK1. Peak enrichment of PDK1 at the synapse, especially in the central portion, was seen at the 3-min, 20-s time point, <3 min after initial conjugate formation. In most conjugates observed, enrichment of PDK1 to the synapse was generally noted to peak within 4 min of T cell-APC contact. PDK1 localization at the synapse subsided in the conjugate shown ~4–6 min after peak enrichment. Visualization of Akt synapse localization essentially exhibited similar kinetics to PDK1, though Akt was noted to have even more pronounced central synapse localization (unpublished data), as has been described by others (39).

We next examined the role of PI3K activity in IL-4 promoter activation and the role of the PH domain in PDK1 localization to the synapse. IL-4 promoter activation by TCR in the presence of CD28 costimulation was sensitive to the PI3K inhibitor LY294002 in the D10 cell line (Fig. 3 C). Akt localization to the immune synapse has been shown to require a functional PH domain (39), albeit several lines of evidence discussed thus far suggest that Akt is not the PI3K-dependent substrate mediating IL-4 activation. We demon-

strated that localization of PDK1 to the immune synapse is also PI3K dependent, as point mutations of critical residues in the PH domain (40) disrupted localization to the T cell-APC interface (Fig. 3 D). In other experiments, treatment of D10 cells with LY294002 also blocked PDK1 localization to the immune synapse (unpublished data).

Activation of the IL-4 promoter element by PDK1 requires cAMP-dependent PKA function

Our original rationale for studying the role of PDK1 in Th2 cytokine activation was its putative role in activation of cAMP-dependent PKA. As noted previously, cAMP and PKA have been shown to exert a positive influence on Th2 cytokine expression in several studies (29, 32). Evidence that PKA participates in PDK1-mediated activation of the IL-4 promoter was initially provided by the observation that the PKA inhibitor H89 could ablate both PDK1 and TCR/CD28-mediated activation of the IL-4 promoter (Fig. 4, A and B). Blockade of TCR/CD28-mediated IL-4 activation by this relatively specific inhibitor is an important observation as it indicates the existence of a PKA-dependent pathway that cannot simply be attributed to kinase overexpression. We evaluated the effects of H89 on calcium flux and Ras activation in D10 cells to rule out nonspecific effects of the inhibitor and, indeed, saw no appreciable effects of H89 on these parameters (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20051715/DC1>). Activation of the IL-4 promoter by PDK1 was also sensitive to the calcineurin inhibitor FK506, providing further evidence that NFAT transcriptional pathways are being targeted by PDK1 (Fig. 4 B).

We next expressed the catalytic subunit of PKA and studied its effects on the IL-4 promoter in D10 cells. Unless stated otherwise, we used the isoform of the human PKA catalytic subunit (PKAc) $\beta 2$ predominantly expressed in human immune tissues (41) for our studies. Use of this isoform was advantageous as it could easily be distinguished from endogenous mouse catalytic subunits as the result of a difference in molecular weight. We have subsequently been able to replicate many of the results discussed here using expression of a mouse PKAc α subunit (unpublished data). As was observed with PDK1, expression of PKAc in D10 cells exhibited robust activation of the IL-4 promoter, with this activation ablated by mutation (T245A) of the putative PDK1 phosphorylation site (Fig. 4 C). This threonine 245 residue is analogous to the critical threonine 197 (T197) residue in the kinase domain of other PKAc isoforms (including PKAc α) (42). Although PKAc T245A generally achieved slightly lower expression levels than wild-type protein (Fig. 4 C), this subtle reduction clearly could not account for the dramatic reduction in IL-4 activation. Even expressed at lower levels, the T245A mutant acts as a potent DN isoform (PKAc DN), completely blocking PDK1-KH-mediated activation of IL-4. In contrast, wild-type PKAc augmented PDK1-mediated activation (Fig. 4 C). Of additional note, expression of wild-type PKAc activates the IL-4 promoter with identical specificity to PDK1 for the NFAT P1 element (unpublished data). Expression of the

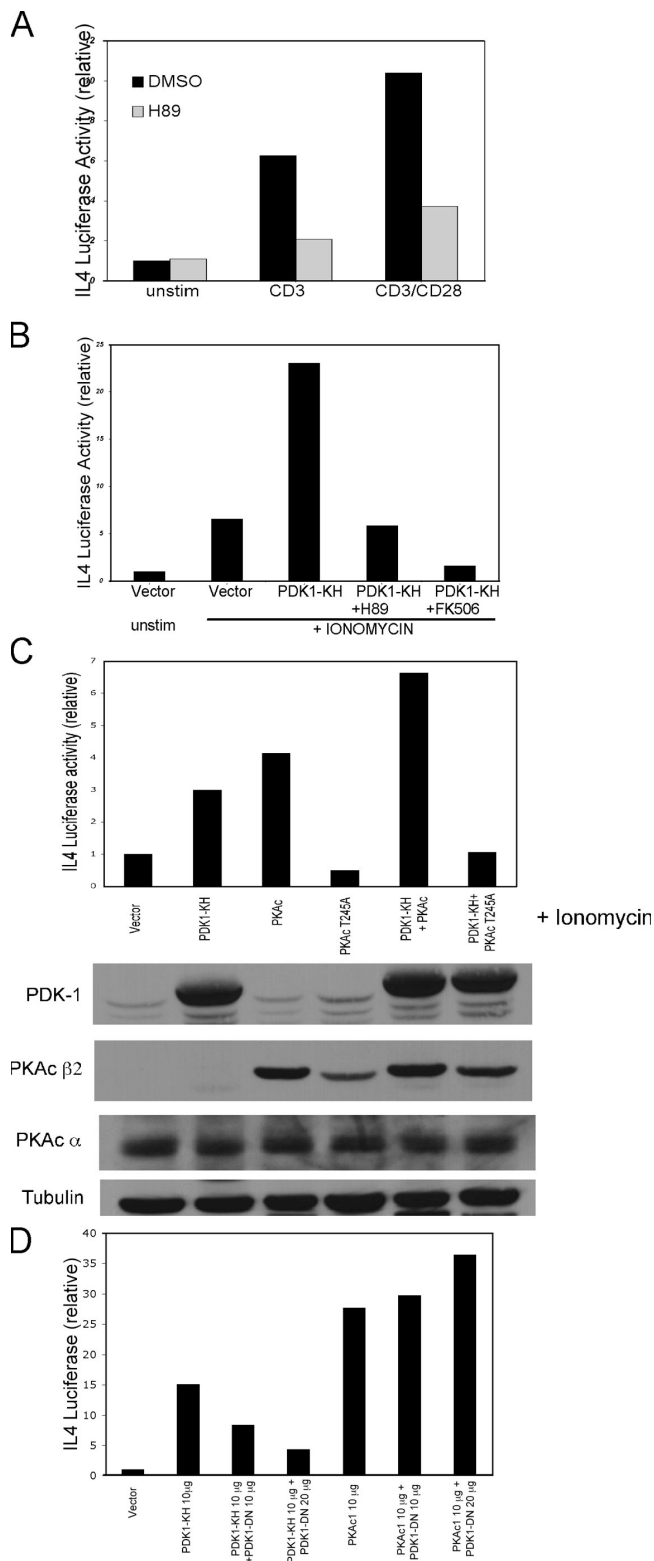


Figure 4. Activation of the IL-4 promoter by PDK1-KH is inhibited by the PKA inhibitor H89. (A) Activation of the IL-4 promoter by CD3/CD28 is inhibited by the PKA inhibitor H89. D10 cells were transfected with IL-4 luciferase and were either unstimulated or stimulated with anti-CD3 antibody or with anti-CD3/CD28 antibodies in the presence of H89

PDK1-DN isoform was able to block activation of IL-4 by PDK1-KH, but not by PKAc (Fig. 4 D). This result suggested that PKAc likely acts downstream of PDK1 in this pathway.

RNAi knockdown of PDK1

The classical mode of PKAc regulation is via dissociation from PKA regulatory subunits in response to stimuli that elevate cAMP levels (43). PKAc also has been shown to undergo regulation by cAMP-independent mechanisms, as it can be maintained in an inactive state via association with IκB proteins (44). We next investigated whether PDK1 regulates PKA via phosphorylation of its kinase domain as well as via physical interaction and colocalization at the immune synapse. RNAi-mediated knockdown of PDK1 expression was used to assess whether PDK1 is responsible for phosphorylation of the critical threonine site in the PKAc kinase domain in D10 T cells. The role of PDK1 in PKA activation has been somewhat unclear because biochemical analyses indicate that PKA is a substrate for PDK1 (21, 22), but analysis of PDK1-deficient embryonic stem cells reportedly reveals normal PKA activity (45). Genetic studies have, however, implicated *ksg1*, the PDK1 fission yeast analogue, as an upstream activator of PKA (46). 2 d after expression of mouse PDK1-specific versus control pooled small interfering RNAs (siRNAs) in D10 cells, we observed a substantial reduction in endogenous PDK1 expression and an associated reduction in phosphorylation of the putative target threonine in endogenous PKAc (Fig. 5 A). The major isoform detected in D10 cells is likely the PKAc α subunit because an α subunit peptide-specific antibody recognizes the protein (Fig. 5 A, third row). Total expression of PKAc α and a control gene, tubulin, were relatively unaffected by the knockdown of PDK1 expression. We next examined the effect of PDK1 knockdown on activation of the critical NFAT P1 element in the IL-4 promoter. Consistent with our earlier data, CD3/CD28-mediated activation of this element is markedly reduced in cells with a PDK1 knockdown (Fig. 5 B). As further evidence that PDK1 can modulate PKA activity, we observed

(10 μM) or a DMSO control. (B) PDK1-KH was cotransfected with IL-4 luciferase into cells that were subsequently stimulated with ionomycin in the presence of vehicle (DMSO), PKA inhibitor H89 (10 μM), or calcineurin inhibitor FK506 (200 ng/ml). (C) PKAc activates the IL-4 promoter with PKAc T245A defective in activation and capable of blocking PDK1-KH-mediated IL-4 activation. PDK1-KH, PKAc β2, and the PKAc β2 T245A mutant were cotransfected with IL-4 luciferase alone or in combination as labeled, with all samples undergoing ionomycin stimulation. Luciferase results are expressed relative to IL-4 Luciferase + vector in the presence of ionomycin (relative activity = 1). Protein expression controls are shown. Transfected PKA is visualized in the second row (PKAc β2) via anti-myc immunoblotting, whereas endogenous PKA is visualized in the third row (PKAc α) using a PKA catalytic α-specific antibody. (D) 10 μg of PDK1-KH or PKAc encoding plasmid vector was expressed alone or with 10 or 20 μg of PDK1-DN and IL-4 Luciferase. All samples were stimulated with ionomycin with results expressed relative to vector control + ionomycin (relative activity = 1).

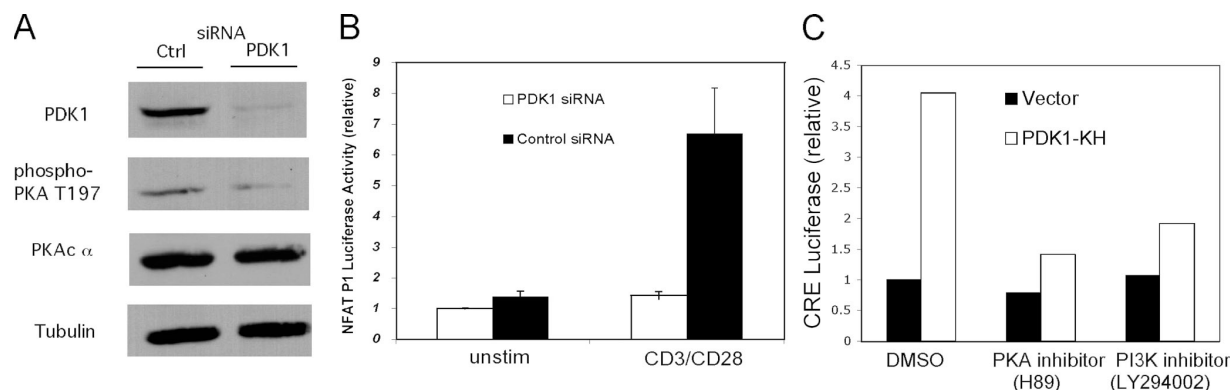


Figure 5. (A) Knockdown of PDK1 expression using RNAi oligos in D10 cells resulted in decreased PKAc α phosphorylation at threonine 197 in the kinase domain. D10 cells were transfected with pooled mouse-PDK1-specific or control pooled RNAi oligos and a nonsignaling human CD16/CD7 fusion construct. On day 2, transfected cells were selected using magnetic bead selection targeting human CD16. Lysates were prepared from 10^6 cells and resolved by SDS-PAGE followed by subsequent immunoblotting. (B) Knockdown of PDK1 expression blocks CD3/CD28-mediated activation of an IL-4 NFAT P1 reporter. D10 cells were cotransfected with an NFAT P1/AP-1 luciferase construct, a nonsignaling

human CD16/CD7 fusion construct, and either pooled mouse-PDK1-specific or control pooled RNAi oligos. At day 2, transfected cells were purified. 10^5 cells were aliquoted in triplicate on a 96-well plate either unstimulated or stimulated with plate-bound anti-CD3 ϵ /soluble anti-CD28 antibody. Results of a representative experiment are expressed as mean luciferase signal with error bars representing standard deviations from the mean. (C) PDK1-KH activates a CRE in a PKA-dependent manner. PDK1-KH or a control vector were cotransfected with a CRE Luciferase reporter in D10 cells. Luciferase activity was subsequently measured from cells treated with DMSO control, H89, or LY294002.

that PDK1-KH stimulates activity of a cAMP-responsive element (CRE) luciferase plasmid and that activation was blocked by H89 (Fig. 5 C). Interestingly, PDK1-mediated activation of CRE luciferase activity is also partially sensitive to LY294002 (Fig. 5 C), suggesting that membrane or synapse induced localization of PDK1 by PI3K may be required for PKA activation.

PDK1 interacts with PKAc and colocalizes with PKAc at the immune synapse

We next sought to define whether PDK1 and PKAc physically associate and also to characterize their relative localization

in T cells. We were able to coimmunoprecipitate a small portion of coexpressed PKAc with PDK1 from 1% NP-40 lysates of D10 T cells (Fig. 6 A). Physical interaction between PDK1 and PKAc, as detected in a yeast two-hybrid assay, has been previously reported (24). Microscopic studies demonstrated that PDK1 and a PKAc-GFP fusion protein colocalize in part at the immune synapse, albeit a significant fraction of PKAc-GFP localizes to the cytoplasm (Fig. 6 B). The PKAc-GFP construct used contained the murine PKAc α subunit fused to GFP (47). We have also performed microscopic studies on the human PKAc β 1 subunit and observed similar partial localization to the synapse (unpublished data).

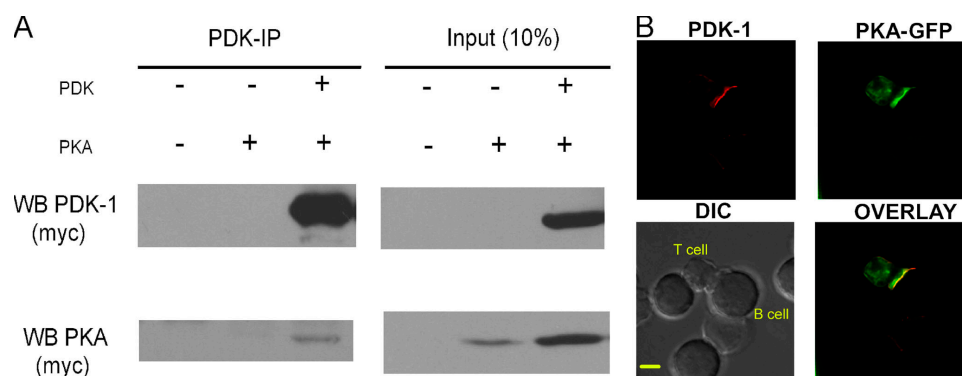


Figure 6. (A) PDK1 interacts with the PKA catalytic subunit. PKAc was expressed alone or in the presence of PDK1. NP-40 (1%) extracts were prepared from 25×10^6 transfected cells. Both proteins were tagged with a myc-epitope. PDK1 was immunoprecipitated with an antibody against the PDK1 carboxy-terminal region and was subsequently immunoblotted for transfected PDK1 and PKA using an anti-myc antibody. 10% of the

amount of extract used for immunoprecipitation was immunoblotted as shown (right). (B) PDK1 and PKA partially colocalize at the immune synapse. D10 cells were transfected with myc-tagged PDK1 and with a PKAc-GFP construct. Cells were allowed to form conjugates with antigen-loaded CH27 cells 16 h later. Cells were fixed and stained with a myc-Cy3 antibody, with microscopy performed as described previously. Bar, 5 μ m.

PKAc is inducibly phosphorylated at threonine 197 in T cells by PDK1

PDK1 has been shown to inducibly phosphorylate several downstream substrates in both PI3K-dependent and independent pathways (28). In T cells, PDK1-mediated phosphorylation of Akt and PKC- θ after CD3/CD28 stimulation has been described previously (5, 48). We sought to determine whether PKA phosphorylation was induced after CD3/CD28 stimulation. Although some degree of basal phosphorylation of the T197 residue of endogenous PKAc α was clearly seen in D10 cells, phosphorylation of the site was augmented at both 30 and 90 min after CD3 and CD28 stimulation (Fig. 7, A and B). A similar up-regulation of phosphorylation was seen for the target PDK1 residues in Akt and PKC- θ . PDK1-mediated phosphorylation of PKA (~ 3.5 fold at 90 min after stimulation) was shown to be PI3K-dependent, as induction of phosphorylation was blocked by incubation with LY2940002 (Fig. 7, A and B). Induction of Akt phosphorylation by PDK1 was PI3K dependent, which was consistent with existing models of Akt activation (Fig. 7 A) (28). Interestingly, although phosphorylation of some PKC family

members has been shown to proceed by a PI3K-independent pathway, induction of PKC θ after CD3/CD28 stimulation was also blocked in part by the PI3K inhibitor. These results argue that PDK1 localization to the synapse is important for phosphorylation of PKA and PKC θ , and not just Akt. It also argues that PI3K-mediated localization of PDK1 to the immune synapse may be critical for activation of multiple substrates in T cells.

Induction of PKA T197 phosphorylation by CD3/CD28 was significantly diminished in cells with a PDK1 expression knockdown by siRNA (Fig. 7 C). As was expected, induction of Akt phosphorylation was similarly blocked, whereas phospho-Erk induction was less affected. This result verified that PDK1 expression was essential for both basal and stimulated phosphorylation of the PKA T197 residue.

DISCUSSION

We have previously reported that PDK1 can help correct the defect in IL-4 cytokine production seen in CD28-deficient primary T cells. Because Akt was unable to achieve similar effects on IL-4 expression, it was hypothesized that PDK1

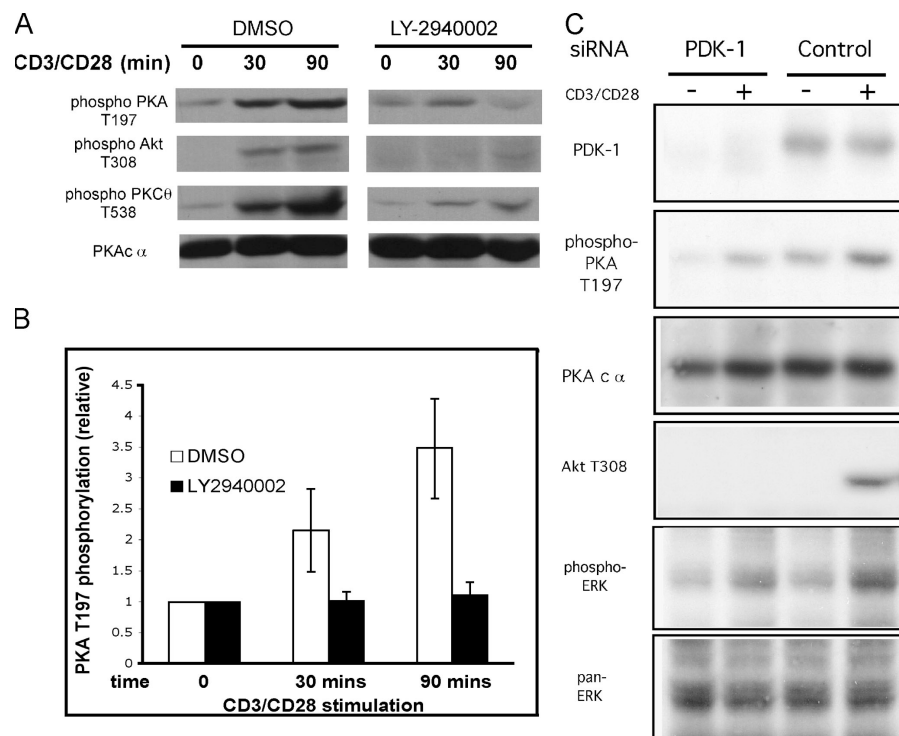


Figure 7. (A) PKA T197 phosphorylation is induced by CD3/CD28 stimulation of D10 T cells. 2×10^6 D10 cells were aliquoted into individual wells on a 24-well plate either unstimulated or stimulated with plate-bound anti-CD3 ϵ /soluble anti-CD28 for the indicated times, in the presence of DMSO or LY2940002 (10 μ M). NP-40 extracts (10^6 cells) were loaded in each lane followed by serial immunoblotting. (B) Quantitation of PKA T197 phosphorylation. PKAc α T197 phosphorylation and total PKAc α signal were quantitated using Image J software. The phospho-T197 signal was divided by total PKA with the baseline T197 index ($t = 0$ min) expressed as 1.0 for both DMSO/LY2940002 samples. Phospho-

T197/total PKA index was calculated for three separate experiments with the graph showing mean result and error bars representing standard deviations. (C) Knockdown of PDK1 expression blocks CD3/CD28-mediated induction of PKA T197 phosphorylation. D10 cells were cotransfected with the CD16/CD7 fusion construct and either pooled mouse-PDK1-specific or control pooled RNAi oligos. At day 2, transfected cells were purified with 10^6 cells from each sample aliquoted on a 24-well plate either unstimulated or stimulated with plate-bound anti-CD3 ϵ /soluble anti-CD28 for 90 min. NP-40 extracts (10^6 cells) were loaded in each lane followed by serial immunoblotting.

targets a different substrate, resulting in IL-4 expression. We have presented studies of PDK1 in the Th2 cell line D10 that reveal molecular mechanisms whereby PDK1 can modulate IL-4 expression. We show that PDK1 activates the IL-4 promoter via a specific NFAT element and that PDK1 localizes to the synapse in a PI3K-dependent fashion. We define PKA as the downstream target of PDK1 action leading to IL-4 expression using several approaches and show that PDK1 is required for phosphorylation of PKA at a critical kinase domain threonine residue in D10 T cells. CD3/CD28 stimulation induces the PDK1-mediated phosphorylation of PKA, with PDK1 and PKA shown to physically interact and partially colocalize at the immune synapse.

Expression of the PDK1-KH molecule in D10 T cells was shown in this report to potentially activate the IL-4 promoter via the NFAT P1 element. One potential concern regarding this result is that the observed effects of PDK1 and PKA may relate to overexpression. We have therefore used DN isoforms of PDK1 and PKA as a complementary approach to block signaling pathways mediated by the T cell receptor and CD28. Both pharmacologic inhibitors of PKA and the DN PDK constructs block IL-4 promoter expression mediated by endogenous proteins. As highly specific inhibitors of PDK1 are not presently available, RNAi experiments have, instead, helped validate a role for PDK1 in phosphorylation of PKAc in T cells, and in regulation of the critical NFAT P1 element in the IL-4 promoter. Although earlier studies in PDK1-deficient ES cells indicated normal phosphorylation of the PKA kinase domain (45), our results suggest that there may be cell type-specific differences in the regulation of PKA phosphorylation.

Activation of an NFAT transcriptional element by PDK1 suggests that this kinase targets multiple signaling pathways in T cells. In separate studies, we have observed that PDK1 can activate NF- κ B pathways, possibly via activation of Akt and PKC- θ (unpublished data). Another recent report confirms that PDK1 targets PKC- θ in the activation of NF- κ B (48). However, PDK1 has multiple other substrates and as such appears capable of regulating multiple transcriptional elements. NFAT transcription factors have been shown to be critical for IL-4 expression in gene knockout studies, with NFATc1 defined as a likely positive regulator of IL-4 but NFATc2 and NFATc3 identified as negative regulatory factors (49–51). Future studies are needed to determine whether PDK1 modulates the occupancy of the NFAT P1 site by NFATc1, as might be predicted from the aforementioned data. It is also worth noting that one study reports that *rel* transcription factors exert a regulatory influence in the region of the NFAT P1 site, though whether PDK1 mediates these effects awaits further studies (52). Lastly, it will be important to determine whether PKA modulates IL-4 expression in Th2 cells via direct phosphorylation of NFAT factors, which is known to take place (53), or via other mechanisms.

Microscopic analysis of PDK1 cellular localization revealed several interesting findings. As has been observed for several critical signaling molecules, PDK1 localizes to the im-

mune synapse in an antigen-dependent fashion, within minutes of conjugate formation. Not surprisingly, colocalization of PDK1 with Akt at the synapse was dependent on PI3K. IL-4 promoter activation by TCR and CD28 stimulation was also PI3K dependent. Of relevance to the signaling pathway described in this report, PDK1 and PKAc colocalize partially at the immune synapse. It will be interesting to examine the relative cellular localization of PKA regulatory subunits and A-kinase anchoring proteins (AKAPs) in T cells. AKAPs have been shown to form the core of large signaling complexes containing PKA subunits, PKC family members, and the PP2A phosphatase in other cell types (54). The role of AKAPs in signaling complexes in T cells is largely undefined as is their role in PDK1-mediated effects on PKA in T cells.

Several lines of evidence were used to establish PKAc as an *in vivo* regulatory target of PDK1. Activation of IL-4 by PDK1 and CD3/CD28 is exquisitely sensitive to PKA inhibition by H89. Additionally, PDK1-mediated activation was blocked by PKA-DN. Common targeting of the NFAT P1 element by both PDK1 and PKA added further support to their combined role in this pathway. In addition to microscopic studies revealing colocalization at the immune synapse, biochemical studies established physical association between PDK1 and PKAc.

Our data have confirmed that the CD3/CD28-mediated induction of PKA phosphorylation and the IL-4 promoter, as well the localization of PDK1 to the synapse, require PI3K activity. Activation of PKAc and a cAMP-responsive reporter by PDK1 were similarly dependent on PI3K. The sensitivity to LY2940002 of PKAc phosphorylation at the PDK1 target residue (Fig. 7, A and B) strongly supports a model in which PKAc must colocalize to the synapse with PDK1 for activation. A direct role for PI3K in PKAc localization to the synapse has not been demonstrated, though PI3K may indirectly influence PKAc localization if interaction with PDK1 plays a role in PKAc synapse localization. Although resolution of this specific issue awaits further investigation, our data clearly indicate that PDK1 and PI3K are required for activation of PKAc in a pathway targeting IL-4.

Several unsettled issues remain with regard to the function of PKA in T cell biology. It is not clear whether a physiologic trigger for cAMP elevation is necessary to allow dissociation of PKAc subunits from regulatory subunits and AKAPs, thereby allowing interaction with PDK1. Some data indicate that TCR and CD28 modulate cAMP levels in some settings (55), though the question has not been addressed adequately in primary undifferentiated T cells or Th2 cells. Another Gs-coupled receptor may be a source of elevated intracellular cAMP levels. An additional question is whether PDK1 plays a direct role in promoting the release of the PKA catalytic subunit from regulatory subunits in a cAMP-independent manner. Of additional note, PKAc has previously been shown to be associated with and be maintained in an inactive state by I κ B proteins, undergoing cAMP-independent release and activation on NF- κ B activation (44). Given the evidence that PDK1 activates NF- κ B

pathways (48), we are investigating whether PDK1 may also exert a regulatory influence on PKAc by promoting this cAMP-independent pathway.

Our studies in the D10 line clearly represent a different cellular system than naive primary T cells where we previously described a role for PDK1 in IL-4 regulation. Our goal with these studies was to define molecular targets of PDK1 with a future aim of analyzing their role in primary T cells. The model shown in Fig. 8 depicts activation of IL-4 by PKA downstream of PDK1. We have not excluded the possibility that PDK1 may exert effects on IL-4 expression by targeting multiple kinases including PKA, and possibly PKC- θ , which has previously been implicated in IL-4 regulation (56) and has been recently shown to be a target of PDK1 action (48). Further studies are needed to delineate the interrelationship of these complex signaling pathways.

MATERIALS AND METHODS

Cells. A rapidly dividing variant of the D10 line was obtained from M. Krummel (University of California, San Francisco, CA). D10 and CH27 cells were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2-mercaptoethanol (50 μ M), as well as human IL-2 (50 U/ml; PBL biomedical laboratories) for the D10 cell line. Cells were restimulated periodically (every 4–6 wk) with mitomycin C-treated splenocytes from B10.BR mice and chicken conalbumin at 100 mg/ml (Sigma Aldrich). CH27 B cells were maintained in RPMI 1640 with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

DNA constructs. Human PDK1 and PKA β catalytic subunit 2 were amplified from Jurkat cDNA by PCR and cloned into the expression vector pCDEF, with a myc tag placed in frame at the 5' end of the cDNA. pCDEF-Akt and Akt-GFP plasmids have been described previously (5, 39). The IL-4–800 promoter plasmid has been described previously (34). Mutants of various plasmids were generated using the QuikChange mutagenesis system (Stratagene). NFAT P1, P1/AP-1, and P0 plasmids were generated by cloning annealed oligonucleotides containing 2X multimers of the relevant sites into the pGL3-Luciferase vector. The CRE luciferase vector (57) and the PKA-GFP plasmid have been described previously (47). L. Kane (University of Pittsburgh, Pittsburgh, PA), C. Vaisse (University of California at San Francisco, San Francisco, CA), and D. Chetkovich (Northwestern University, Chicago, IL) provided plasmids.

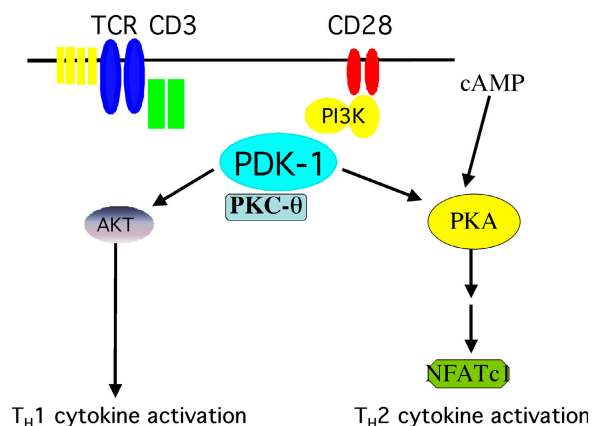


Figure 8. Model for activation of the IL-4 promoter by PDK1 and PKA in Th2 cells.

Antibodies. Antibodies were obtained from the following sources: anti-PDK1 used for Western blotting (BD Transduction Laboratories), anti-PDK1 for immunoprecipitations (Upstate Laboratories), anti-phospho PKA (Biosource International), pan-PKA (Upstate Laboratories), anti-myc (Cell Signaling), rabbit anti-hamster IgG (Sigma-Aldrich), anti-CD3 ϵ 2C11 (BD Biosciences), anti-CD28 37.51 (murine ascites preparation), and anti-human CD16-FITC (BD Biosciences).

Transfection/stimulations. D10 cells were resuspended at $35\text{--}50 \times 10^6$ cells/ml and were electroporated with relevant plasmids in a Gene-Pulser (Bio-Rad Laboratories) at 250V, 950 μ F. 20 μ g of each plasmid was transfected in such experiments (unless otherwise noted). Cells were cultured in 10 ml complete D10 medium and IL-2 at 50 U/ml overnight, before subsequent luciferase assays, biochemical analyses, or microscopy.

Luciferase assays. Cells were resuspended at 2×10^6 cells/ml in complete D10 medium without IL-2. 50 μ l of cells was aliquoted into round bottom 96-well plates with 50 μ l of media containing the relevant stimulus. Ionomycin, H89, and LY294002 were purchased from CalBiochem. FK506 was obtained from the UCSF pharmacy. For plate-coated CD3 stimulation, 96-well plates were coated overnight at 4°C with goat anti-hamster IgG (10 μ g/ml in 0.1 M Carbonate Buffer), with subsequent washing and incubation with anti-CD3 antibody (1 μ g/ml in medium) at 37°C for 2 h. Wells were washed once with complete D10 medium before addition of cells. Anti-mouse CD28 antibody was added where indicated (1:500 in media). After 6 h, 100 μ l of cells was permeabilized with 11 μ l luciferase harvest buffer (10% Triton X-100, 1 mM DTT and 0.2 M KPO₄, pH 7.8) and incubated for 5 min at room temperature. Samples were transferred to an opaque 96-well plate and 100 μ l of luciferase assay buffer was added (10 mM ATP, 20 mM MgCl₂, and 0.2 M KPO₄, pH 7.8). Luciferase activity was determined with a 10-s reading after injection with 50 μ l of 1 mM luciferin, in either a MicroLumat luminometer or a Mithras LB 940 luminometer (EG&G Berthold).

Cell extract preparation/immunoprecipitations/SDS PAGE. Cells were washed in PBS and lysed on ice in 1% NP-40 buffer with 150 mM NaCl, 20 mM Tris pH 7.5 with protease inhibitors. After 20 min on ice, samples were centrifuged for 5 min at 13,000 revolutions/min in a Sorvall Biofuge Fresco at 4°C with postnuclear supernatants saved. Extracts were mixed with reducing sample buffer and boiled for 5 min before loading on a SDS polyacrylamide gel (10% unless stated otherwise). For immunoprecipitations, cell extracts were incubated with rotation for 4 h at 4°C with the precipitating antibody and 20 ml of protein G-sepharose bead slurry. Beads were subsequently centrifuged for 5 s before each of four washes with 1% NP-40 lysis buffer. Beads were resuspended in reducing buffer and boiled for 5 min before loading on a gel. Ras activation assays are described in the supplemental Materials and methods section (available at <http://www.jem.org/cgi/content/full/jem.20051715/DC1>).

Immunoblotting. After resolution of proteins on an SDS-polyacrylamide gel, proteins were transferred to a PVDF membrane (Millipore) with a semidry blotting apparatus. Membranes were blocked in 3% BSA in 250 mM NaCl, 20 mM Tris, pH 7.5, 0.05% Tween 20 (TBS-T). Primary antibody was incubated in TBS-T, 1% BSA at a 1:1,000 dilution (unless stated otherwise) either overnight at 4°C, or for 1 h at room temperature. Blots were washed four times for 5 min in TBS-T before incubation with the relevant secondary antibody-horseradish peroxidase (HRP) conjugate at 1:10,000 for 30 min at room temperature. After four 10-min washes in TBS-T, membranes were developed with enhanced chemiluminescence (GE Healthcare) and subsequent autoradiography. Blots were stripped with 67 mM Tris pH 6.8, 2% SDS, 0.1% 2-mercaptoethanol before incubation with other antibodies. Individual protein bands were quantified following autoradiography with Image J 1.33u software (freeware; National Institutes of Health).

Immunocytochemistry/microscopy. Cells were incubated overnight after transfection and live cells were isolated on a lympholyte (Cedarlane Laboratories)

gradient. CH27 cells were incubated overnight in complete CH27 medium with chicken conalbumin protein (100 $\mu\text{g}/\text{ml}$). Equal numbers of D10 and CH27 cells (4×10^6 cells total in 1 ml media) were mixed and centrifuged at 1,200 g for 1 min with subsequent incubation at 37°C for 20 min. Medium was aspirated followed by gentle resuspension of conjugates in PBS, 1% BSA. 10^5 cells were spotted on poly-L-lysine-coated slides followed by fixation of cells in 4% formaldehyde for 20 min, one wash with PBS 0.2% BSA, and permeabilization with 0.2% Triton X-100. Cells were blocked for 10 min with PBS, 1% BSA, 2% normal mouse serum. Cells were stained with anti-myc Cy3 at 1:50 in blocking buffer. Cells were washed three times for 5 min and mounted with coverslips with polyvinyl alcohol mounting medium.

Images were collected on a Marianis system with a Sensicam cooled CCD camera (Cooke) attached to an Axiovert microscope with a Zeiss Plan-Apochromat 63X/1.4 NA oil objective (Carl Zeiss MicroImaging, Inc.). 15–20 fluorescence and differential interference contrast (DIC) images were collected per stack. Data stacks (z-axis) were subjected to constrained iterative deconvolution using an Apple Macintosh G5 computer equipped with Slidebook software (Intelligent Imaging Innovations). Blinded analysis of PDK1 localization was performed by identifying conjugates with Akt localized at the immune synapse and by subsequent scoring of PDK1 localization either as exclusively at the synapse, partially at the synapse, or completely excluded from the synapse.

For time lapse fluorescence microscopy, we used a modified Axiovert 200M microscope with a Plan Neofluar 40 objective (Carl Zeiss MicroImaging, Inc.). The microscope was fitted with dual excitation and emission filter wheels and a Photometrics Coolsnap-HQ camera. Metamorph was the imaging and control software (Universal Imaging). Antigen-loaded CH27 cells were plated into coverslip wells (Nunc) and were maintained at 37°C on the heated stage of the microscope. PDK1-GFP transfected D10 cells were added into APC-containing coverslip wells. Data were collected for 20 min with 40-s intervals for DIC and green fluorescence images at the midcell z-section. Time point before the initial contact was observed between T cell and APC was set to “0.”

RNAi experiments. D10 cells were electroporated with mouse PDK1-specific pooled siRNAs or control pooled siRNAs (250 nM final concentration; Dharmacon). 44 h after transfection, live cells were isolated via centrifugation in a lympholyte gradient and were stained with anti-human CD16-FITC. Cells were analyzed by FACS to verify equivalent transfection efficiencies between samples. The remainder of cells were purified using the MACS magnetic bead cell isolation system using anti-FITC conjugated beads (Miltenyi Biotec). 2×10^5 cells were analyzed by FACS to ensure enrichment of transfected cells to >90% of total. 10^6 cells were resolved by SDS-PAGE and subjected to serial immunoblotting with several antibodies.

Online supplemental material. Fig. S1 demonstrates that the PDK1 S241A mutant acts as a DN isoform and can block induction of Akt phosphorylation after CD3/CD28 stimulation of D10 T cells. Fig. S2 evaluates the specificity of the H89 inhibitor, demonstrating no appreciable effects on calcium flux and Ras activation in D10 cells. Figs. S1 and S2 as well as a supplemental Materials and Methods section are available at <http://www.jem.org/cgi/content/full/jem.20051715/DC1>.

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